AD_____

Award Number: W81XWH-11-1-0309

TITLE: Targeting the Human Complement Membrane Attack Complex to Selectively Kill Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, MD

CONTRACTING ORGANIZATION:
Johns Hopkins University, East Baltimore Campus
Baltimore, MD 21205

REPORT DATE: December 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2014	Final	30 SEP 2011 - 29 SEP 2014

4. TITLE AND SUBTITLE

Targeting the Human Complement Membrane Attack Complex to Selectively Kill Prostate Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0309

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Samuel R. Denmeade

E-Mail: denmesa@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University, East Baltimore Campus
Department of Pharmacology and Molecular Sciences
725 North Wolfe Street, WBSB 302
Baltimore, MD 21205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT The goal of this proposal is to develop a strategy to redirect the toxicity of a human protein to sites of prostate cancer. Our hypothesis is that the human C5 complement protein can be modified to a form that is not activated by the normal complement pathways but is instead activated by the prostate cancer specific protease PSA. This strategy would allow us to selectively unleash the potent cytolytic activity of the human complement Membrane Attack Complex (MAC) within sites of metastatic prostate cancer while sparing normal host tissue due to the finely tuned regulation of complement activity in the circulation. A series of PSA-activated modified C5 proteins were generated and characterized for PSA activation. While initial studies suggested that wild type C5 was stable in the presence of PSA, further characterization demonstrated that both the wild type and modified C5 proteins were extensively cleaved and degraded by PSA. Thus, while PSA could cleave the engineered cleavage site within the modified protein, it also cleaved the protein at multiple off-target sites. PSA was able to inactivate complement fixation and MAC formation through degradation of wild type C5 suggesting a potential role for PSA in regulation of complement activity within prostate cancer. Based on the finding of PSA degradation of C5, we are now exploring whether alternative proteases selectively overexpressed in prostate cancers such as human glandular kallikrein 2, TMPRSS2 and fibroblast activation protein (FAP) can be targeted to activate a modified C5 protein using the same strategy we have outlined for the PSA studies. These new modified C5 proteins will be evaluated for appropriate cleavage at the engineered site and for selective toxicity.

15. SUBJECT TERMS
Prostate cancer, protoxin, prodrug, PSA, FAP, complement, targeted therapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
Introduction……..……...…..	**4**
Body……..	**5**
Key Research Accomplishments…….……..	**7**
Reportable Outcomes……	**7**
Conclusion……	**8**
References……	**9**

Introduction: The goal of this proposal is to develop a strategy to redirect the toxicity of a human protein to sites of prostate cancer. Our original hypothesis is that the human C5 complement protein can be modified to a form that is not activated by the normal complement pathways but is instead activated by the prostate cancer specific protease PSA. This strategy would allow us to selectively unleash the potent cytolytic activity of the human complement Membrane Attack Complex (MAC) within sites of metastatic prostate cancer while sparing normal host tissue due to the finely tuned regulation of complement activity in the circulation. One advantage of this strategy is that activation results in generation of the MAC within the extracellular fluid (ECF) bathing the prostate cancer cells leading to the lytic death of PSA-producing prostate cancer cells as well as a significant bystander effect and killing of non-PSA producing cancer cells, endothelial cells and fibroblasts that are also bathed in PSA in the ECF. This strategy is predicated by the observation that PSA is completely INACTIVE in the blood of prostate cancer patients. Thus, our idea was that the PAC5 proteins could be administered systemically without activation in the blood of patients with high serum PSA levels. Over the first two years of the project we generated PAC5 proteins as described in Aims 1 and 2 below. We learned that these PAC5 proteins could be correctly processed by PSA. However, over the course of these studies it became clear that C5 was readily degraded by PSA. In further studies we learned that PSA degraded other complement proteins including isoforms of C3. These results suggested a potential role for PSA's proteolytic activity as an immunomodulator in both human seminal fluid as well as in prostate cancer. These findings were described published in the Journal of Immunology (Manning ML, Williams SA, Jelinek CA, Kostova M, Denmeade SR. Proteolysis of Complement Factors iC3b and C5 by the Serine Protease Prostate-Specific Antigen (PSA) in Prostatic Fluid and Seminal Plasma. J Immunol. 2013 Mar 15;190(6):2567-74).

These results also suggested that our approach to modify C5 into a PSA-activatable form would likely not work due to the ability of PSA to degrade and inactivate C5. Therefore, we refocused on our original goal which was to develop a strategy to redirect the toxicity of a human protein to sites of prostate cancer. We opted to try to modify a different human protein as a potential PSA activated protoxin. The alternative protein we selected was Granzyme B. Granzyme B is a protease that is produced by T cells and is a major factor in T cell mediated killing of target cells. Granzyme B is produced as an inactive zymogen. It has an N-terminal dipeptide that mus be proteolytically removed to activate the protease activity. Once activated granzyme B enters the cytoplasm via a perforin-mediated uptake mechanism, it cleaves caspases.

GZMB activation in vivo is a two-step process that requires proteolytic processing to remove the N-terminal dipeptide pro-piece followed by cell binding and cell entry that is facilitated by Perforin. Our goal is to generate a modified GZMB toxin that also requires a two-step activation process to produce a toxin that is specifically toxic to prostate cancer cells but non-toxic to normal cells. **First, we will modify the N-terminus of GZMB and replace the native N-terminal dipeptide with a 6 amino acid peptide recognized as a substrate by PSA.** Human GZMB is proteolytically activated on the N-terminus by removal of a GE peptide thus allowing the protein to fold into its active conformation. Because it has been shown that other peptides can be used to modify the proteolytic activation of GZMB (27), we propose to make PSA activated mutants by replacing the GE peptide with an array of known PSA substrates (19) **(Figure 1).** These mutants will be tested for their specificity and potency against PSA positive/negative cells in conjunction with the PSMA binding urea targeting studies.

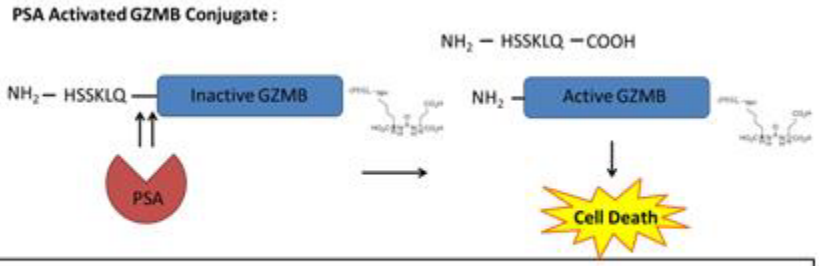

Figure 1: Cartoon depicting the activation WT (top) and PSA activated (bottom) GZMB urea conjugate.

Second, to achieve cell binding and uptake, we propose to link a PSMA binding urea to the C-terminus of recombinant GZMB. This will be done by introducing a reactive cysteine as the C-terminal amino acid (as the 248th AA in the WT protein). Maleimide-PEG-NHS linkers of various lengths will be linked to the free amine of the PSMA urea in order to covalently link the compound to the C-terminus of GZMB. The C-terminus was chosen based on the design of successful (*in vitro* and *in vivo*) antibody linked GZMB based immunotoxins (28-30)**.** We hypothesize that linking a PSMA binding urea to the C-terminus will facilitate its delivery and internalization by PSMA expressing prostate cancer cells. Initially, we will synthesize a proteolytically activated form of GZMB and assess the effect of the conjugation on cell targeting and potency of the protein.

This dual-regulated GZMB pro-drug differs from other published GZMB-based targeted agents as it does not use an antibody or growth factor as its mode of targeting. This decreases the likelihood that the construct will form novel antigenic epitopes seen in fusion proteins. Also in contrast to GZMB fusion proteins, our GZMB pro-drug will be enzymatically inactive until it reaches the tumor microenvironment. Both of these are advantages that will increase specificity of the drug and decrease unwanted toxicity and immunogenicity.

Proposed Mechanism of Action: A PSA activated GZMB-Urea construct will be given IV and will be initially inactive in the bloodstream due to the lack of enzymatic PSA. Once it reaches the prostate tumor microenvironment, active PSA will cleave off the N-terminal inhibitory peptide of the pro-drug. Active GZMB-urea will then bind to PSMA on the cell membrane and be internalized via endocytosis. Once in the cytoplasm of the cell, active GZMB will cleave a myriad of pro-apoptotic substrates triggering a cascade of events leading to death of the tumor cell. This construct, being of human origin will likely not generate an immune response and will have minimal to no effect on non-cancerous tissue, as the pro-drug requires both PSA and PSMA to be functional, **(Figure 2).**

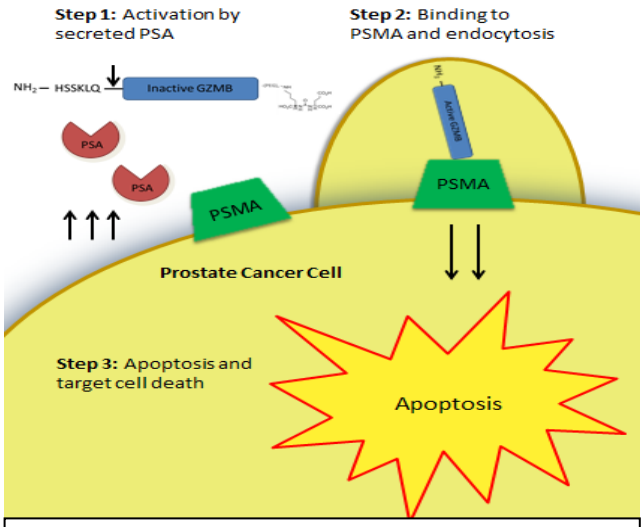

Figure 2: Mechanism of action of PSA-activated GZMB-PSMA urea conjugate.

Progress over the 2013-2014 Funding Period:

Based on the revised plan we pursued two new specific aims over the 2013-2014 funding period.
Specific Aim 1 (Task 3 in Revised SOW): Identify the optimal PSA-activated GZMB containing a PSA substrate as the pro-peptide. The gene for human GZMB has been cloned into a mammalian expression cDNA vector. The WT inhibitory/activation dipeptide Gly-Glu will be mutagenized to generate GZMB proteins containing PSA substrates differing in efficiency and specificity for PSA hydrolysis compared to other proteases.
Specific Aim 2 (Task 4 in Revised SOW): Enhance the targeting of a PSA activated GZMB with a PSMA binding urea. PSA activated pro-GZMB constructs with acceptable stability in serum that are efficiently cleaved by PSA (preferably >50% activation within 4 hr. incubation) will be further modified by coupling to the PSMA-urea inhibitor.

In order to develop a reliable expression purification system of rGZMB, we utilized the methods of expressing the protein described in Gerhmann (27) et al. and purified the protein based on its unusually high pI of 9.5. This protein was expressed with an enterokinase (EK) inactivation peptide on the N-terminus in HEK-293T cells. We purified the secreted protein using ion exchange chromatography at pH 7.4. To assess the activity of the protein with and without EK present, we used a fluorescent GZMB-specific proteolysis assay. **Figure 3A** shows EK specific proteolytic activation of a purified rGZMB mutant containing a cysteine on the C-terminus (C248). This protein had no activity without pre-incubation with EK. This result demonstrates that the N-terminus of GZMB can be modified with alternative peptide sequences and still yield active enzyme after processing.

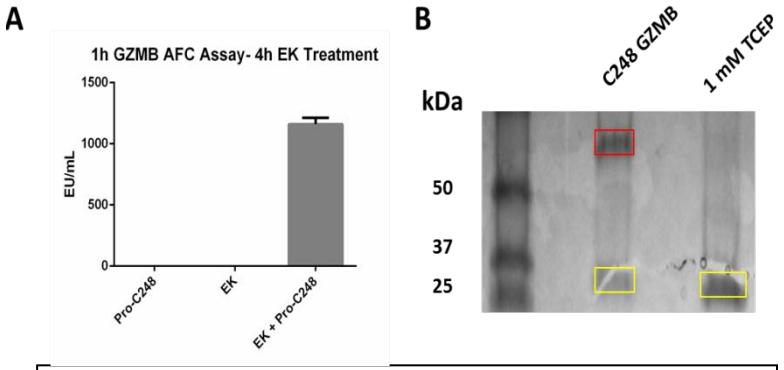

Figure 3: A. EK activated C248 (EK-C248) GZMB is enzymatically active upon EK treatment. **B**. EK-C248 forms reducible disulfide-linked dimers.

We next confirmed the presence of a C-terminal reactive cysteine on C248 following ion exchange purification. Purified C248 rGZMB was pretreated with buffer or 1 mM of the non-thiol reducing agent TCEP at RT. A vast majority of the non-reduced sample existed in a disulfide linked dimeric form (native GZMB exists in a monomeric form) that was completely removed by rapid TCEP reduction **(Fig 3B).**

Once the reactive, functional GZMB mutant EK-C248 mutant was expressed, purified, and characterized, we determined whether a PSMA binding urea could be successfully coupled to this protein. To do this, we reacted a maleimide-linked PEG-8 urea (MU8), with EK-C248 GZMB. We then digested the protein with EK and purified the protein conjugate using ion exchange chromatography. Unlike the reactive, dimer-forming, C248 mutant, MU8

coupled GZMB (GZMB-MU8) did not form disulfide dimers in solution suggesting complete coupling of the maleimide to the free thiol group (data not shown). To characterize the functionality of the protein-drug conjugate, we incubated the purified GZMB-MU8 construct with PSMA-rich LNCaP cell lysates with the PSMA substrate NAAG. Because NAAG cleavage by PSMA yields N-acetyl-aspartate and free glutamate, PSMA activity was determined using a free glutamate detection kit (Life Technologies). GZMB-MU8 was given in a series of increasing doses in order to establish potency. GZMB-MU8 inhibited PSMA activity when incubated with the lysates at 37 degrees Celsius for 4 hours with an IC_{50} of approximately 700 nM **(Figure 4A).** This result suggests that GZMB-MU8 is able to bind to the catalytic site of PSMA. We next evaluated the enzymatic activity of GZMB-MU8 in comparison to GZMB-C248. Remarkably, urea-conjugated GZMB-MU8 had a 20 fold higher enzymatic activity per mg protein than the non-conjugated C248 mutant **(Figure 4B).** We will further investigate this result in order to determine the effect of urea coupling on the enzymatic activity of GZMB.

Finally, we wanted to assess the feasibility of constructing a PSA activated GZMB mutant. To do this we replaced the EK inhibitory peptide with HSSKLQ, a highly specific PSA substrate previously described by the Denmeade lab (9). The mutant (PSA-C248) was expressed and purified in the manner described above. This protein was incubated with buffer or active human PSA and its enzymatic activity was characterized using the fluorescent GZMB assay. In the absence of PSA, PSA-C248 GZMB had a low level of baseline activity. However, this activity increased 4 fold when PSA was added **(Fig. 5).** PSA alone had no activity in this assay.

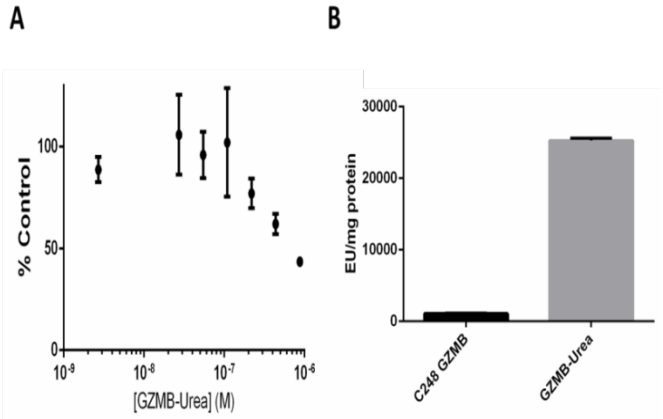

Figure 4: A. GZMB-MU8 binds and inhibits PSMA. **B**. GZMB-MU8 is 20-fold more active than the C248 mutant

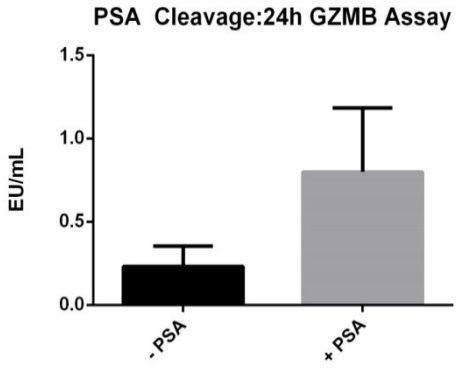

Figure 5: PSA-C248 enzymatic activity increases with PSA.

Key Research Accomplishments:

- Putative PSA-activated modified C5 proteins were generated and characterized for PSA cleavage
- Homology modeling of the modified C5 proteins was performed
- A C5 mutant designated "PAC5-7" was created and verified to be PSA-cleavable by mass spectroscopy.
- PAC5-7 displayed an unexpected cleavage pattern when treated with PSA; that is, additional cleavage sites were present. Further studies determined these sites were carried over from wild type C5. It would be a time intensive process to engineer these sites out, with no guarantee of success.
- PSA found to inactivate complement activation through cleavage of C5.
- PSA plays a role in the degradation of complement factor C3.
- PSA-activated granzyme B protein was produced and shown to be activated by PSA
- Modified Granzyme B protein containing PSMA-inhibitor urea was generated and demonstrated to bind to and inhibit PSMA

Reportable Outcomes:

- Graduate student Michael Manning received a pre-doctoral training award from the DOD PCRP (W81XWH-10-PCRP-IDA).
- Graduate student Michael Manning receive his Ph.D. degree (Spring 2012) from the Johns Hopkins University School of Medicine, Department of Pharmacology and Molecular Sciences for this work.
- Graduate student Oliver Rogers received support for his Ph.D. training from the Johns Hopkins University School of Medicine, Department of Pharmacology and Molecular Sciences for this work.
- Manning ML, Williams SA, Jelinek CA, Kostova M, Denmeade SR. Proteolysis of Complement Factors iC3b and C5 by the Serine Protease Prostate-Specific Antigen (PSA) in Prostatic Fluid and Seminal Plasma. Immunol. 2013 Mar 15;190(6):2567-74.
- Manning ML, Kostova M, Williams SA, Denmeade SR. Trypsin-Like Proteolytic Contamination of Commercially Available PSA Purified from Human Seminal Fluid. Prostate. 2012;72:1233-8.
- Presentations:

1. **Manning ML**, Denmeade SR. (2012) Prostate-specific antigen (PSA) is an immunomodulator. Johns Hopkins Prostate Research Day, Baltimore, MD, February 25, 2012.
2. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) is an immunomodulator. NIH National Graduate Student Research Conference, Bethesda, MD, October 17, 2011.
3. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) is an immunomodulator. Cancer Immunology and Immunotherapy – NCI, Bethesda, MD, September 22, 2011.
4. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) is an immunomodulator. Johns Hopkins Pharmacology Research Retreat, Baltimore, MD, September 10, 2011.
5. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) is an immunomodulator. SKCCC Fellow Research Day, Baltimore, MD, May 17, 2011.
6. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) cleavage and release of activated complement proteins from the cell surface. Johns Hopkins GSA Poster Competition, Baltimore, MD, April, 2011.
7. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) cleavage and release of activated complement proteins from the cell surface. Multi-Institutional Prostate Cancer Program Retreat, Ft. Lauderdale, FL, March 22, 2011.
8. **Manning ML**, Denmeade SR. (2011) Engineering a Cytolytic Human Protein into a Novel Prostate Cancer Protoxin. Department of Defense Innovative Minds in Prostate Cancer Today Conference, Orlando, FL, March 10, 2011.
9. **Manning ML**, Denmeade SR. (2011) Prostate-specific antigen (PSA) cleavage and release of activated complement proteins from the cell surface. Johns Hopkins Prostate Research Day, Baltimore, MD, February 5, 2011.

Conclusion:

Substantial progress was made in the first two years of the grant past year to complete the tasks outlined in Aims 1 and 2 of the grant. Unexpectedly, these studies led us to explore a potential role for PSA in modulating the immune system through degradation of two key complement proteins, C3 and C5. We demonstrated that PSA cleavage of these proteins inactivates their function and ability to generate the membrane attack complex. We are continuing to explore these findings to determine if PSA may play a role in promoting prostate tumor growth through inhibition of complement mediated tumor cell destruction.

However, these results suggest that the development of a PSA-activated C5 protein toxin will be problematic based on degradation of the C5 protein by PSA. Therefore, for the last year of funding we refocused on our original goal which was to develop a strategy to redirect the toxicity of a human protein to sites of prostate cancer. We opted to try to modify a different human protein as a potential PSA activated protoxin. The alternative protein we selected was Granzyme B (GZMB). GZMB is a protease that is produced by T cells and is a major factor in T cell mediated killing of target cells. GZMB is produced as an inactive zymogen. It has an N-terminal dipeptide that mus be proteolytically removed to activate the protease activity. Once activated granzyme B enters the cytoplasm via a perforin-mediated uptake mechanism, it cleaves caspases. GZMB activation in vivo is a two-step process that requires proteolytic processing to remove the N-terminal dipeptide pro-piece followed by cell binding and cell entry that is facilitated by Perforin. Our goal is to generate a modified GZMB toxin that also requires a two-step activation process to produce a toxin that is specifically toxic to prostate cancer cells but non-toxic to normal cells. First, we proposed to modify the N-terminus of GZMB and replace the native N-terminal dipeptide with a 6 amino acid peptide recognized as a substrate by PSA. Second, to achieve cell binding and uptake, we proposed to link a PSMA binding urea to the C-terminus of recombinant GZMB. Over the 2013-2014 funding period we have produced a PSA-activated granzyme B protein d and shown it to be activated by PSA. We have also generated a modified Granzyme B protein containing PSMA-inhibitor urea and demonstrated its ability to bind to and inhibit PSMA. We are proceeding to make additional proteins to identify the optimal PSA activation sequence and will generate a GZMB protein containing both the PSA activation sequence and the PSMA binding inhibitor to determine if the dual targeting can effectively and selectively kill prostate cancer cells.

References:

1. Ogata, R.T. and P.J. Low, *Complement component C5: engineering of a mutant that is specifically cleaved by the C4-specific C1s protease.* J Immunol, 1995. **155**(5): p. 2642-2651.
2. Kopp, J. and T. Schwede, *The SWISS-MODEL Repository of annotated three-dimensional protein structure homology models.* Nucl. Acids Res., 2004. **32**(suppl_1): p. D230-234.
3. Fredslund, F., et al., *Structure of and influence of a tick complement inhibitor on human complement component 5.* Nat Immunol, 2008. **9**(7): p. 753-760.
4. He, T.-C., et al., *A simplified system for generating recombinant adenoviruses.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(5): p. 2509-2514.
5. Rozanov, D.V., et al., *Interference with the complement system by tumor cell membrane type-1 matrix metalloproteinase plays a significant role in promoting metastasis in mice.* Cancer Res., 2006. **66**(12): p. 6258-63.
6. Frade, R., et al., *Procathepsin-L, a proteinase that cleaves human C3 (the third component of complement), confers high tumorigenic and metastatic properties to human melanoma cells.* Cancer Res., 1998. **58**(13): p. 2733-6.
7. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res., **25**: p. 3389-3402.
8. Garin-Chesa, P., et al., *Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers.* Proc Natl Acad Sci 1990;**87**: p.7235-39.
9. Scanlan, M.J., et al., *Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers.* Proc Natl Acad Sci 1994;**91**: p.5657-61.
10. Park J.E., et al., *Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts.* J Biol Chem 1999;**274**: p.36505-12.
11. Aggarwal, S., et al., *Fibroblast Activation Protein Peptide Substrates Identified from Human Collagen I Derived Gelatin Cleavage Sites.* Biochemistry 2008;**47**: p.1076-86.
12. Lebeau, A.M., et al., *Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin.* Mol Cancer Ther. 2009; ;**8**(5): p.1378-86.